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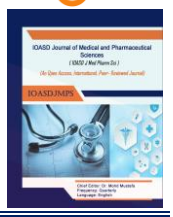

Bio production of Indole 3-Acetic Acid by *Bacillus pumilus* DS5 isolated from agricultural field soils of Andhra Pradesh

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ABSTRACT

Bacillus pumilus DS5, a plant growth-promoting bacterium, was isolated from soil samples taken from agricultural fields in the vicinity of Guntur, Andhra Pradesh. The isolate was characterized using morphological, biochemical, and physiological methods to determine its taxonomic position. The bacterium was identified by sequencing its 16S rRNA and depositing the sequences in GenBank (NCBI) under accession number (MG870111). The strain was found to produce Indole Acetic Acid (IAA) in YEM broth medium, and the production of IAA was further studied in optimization experiments. The effects of incubation period, pH, temperature, vitamins, carbon, and nitrogen sources on IAA production were investigated. The maximum IAA production of 105 µg/ml was observed after 48 h of incubation using 0.1% L-tryptophan at pH 7.0. Additionally, the effects of different carbon and nitrogen sources on IAA production were examined. Sucrose and yeast extract were found to be the best carbon and nitrogen sources, respectively, for maximum production of IAA. These results suggest that Bacillus pumilus DS5 could be a useful bacterium for promoting plant growth promoting studies.

Keywords: Indole acetic acid (IAA), *Bacillus pumilus*, Plant growth promoters.

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INTRODUCTION

Rhizosphere microorganisms known as Plant Growth Promoting Rhizobacteria (PGPR) have been identified as a subset of microorganisms that can increase plant growth and sometimes provide biological control against plant pathogens, aid in nutrient cycling, and support seedling establishment. The successful inoculation of agricultural crops with biocontrol PGPR requires sufficient delivery of inoculum to the target, cost-effective production of large quantities of microorganisms, extended shelf life, and the development of a convenient formulation. The efficacy of the inoculum largely depends on the chosen formulation and delivery method for microbial inoculants.

Plant growth-promoting rhizobacteria (PGPR) are a group of free-living bacteria that colonize the rhizosphere and contribute to increased growth and yield of crop plants (Kloepper and Schroth, 1978). The ability of PGPR to promote growth may be highly specific to a particular species, cultivar, soil, and genotype (Haung and Annapurna, 2004). Therefore, it is essential to study the native bacterial population, their distribution, and diversity. Thus, studies on region-specific microbial strains that can be used as potential plant growth promoters under specific environmental conditions have gained importance.

Application of biopolymers such as chitin and chitosan to farming soils has resulted in plant growth promotion and disease control. The addition of composts to soil has also increased the incidence of PGPR in the rhizosphere and decreased the disease incidence. The production of Indole-3-acetic acid (IAA), siderophores, and phosphate solubilization are important plant growth-promoting traits for rhizobacteria (Kumar and Ram, 2014).

Many microorganisms in the rhizosphere are known to produce plant hormones, such as auxins and gibberellins, that stimulate plant growth and development. The relationship between plant growth-promoting bacteria (PGB) and microbial species has been extensively studied (Klopper and Schroth, 1978; Hurek and Reinhold-Hurek, 2003). Bacillus pumilus and B. licheniformis are known to produce GA3 and auxins, and these plant growth-promoting substances are widely

used in agriculture, nurseries, and tissue culture (Shukla et al., 2005; Gutierrez-Manero, 2001.

Plant growth hormones are essential for modern agriculture, and a particular PGPR may affect plant growth and development through one or more mechanisms, which may be region-specific. The diverse species of microorganisms in the rhizosphere also exhibit variations in growth and yield. Microorganisms in the rhizosphere are dependent on specific conditions and regions, and they play an important role in plant growth and yield enhancement (Kumar and Ram, 2012). PGPR are active at various levels of biodiversity and play important roles in ecosystem processes such as biological control of plant pathogens, nutrient cycling, and seedling establishment (Kloepper et al., 1991; Glick, 1995).

More than 80% of the bacteria isolated from rhizospheric soils are capable of producing IAA, which is derived from the precursor tryptophan (Khalid et al., 2004; Patten and Glick, 1996). Various tryptophan-dependent and tryptophan-independent pathways in plants have been described in bacteria and fungi (Patten and Glick, 2002). The genus Bacillus stands out as one of the main genera of PGPR used to promote plant growth. Bacillus thuringiensis is potential for controlling plant parasitic nematodes in crop plants, insect vectors of diseases, and the production of bio-insecticides (Silva et al., 2011; Soccol et al., 2009) for use in agriculture. In this study, we screened ten isolates from agricultural field soils in Guntur for their plant growth-promoting characteristics, including IAA production, to exploit them as bio-inoculants. Bacillus pumilus DS5, which showed copious production of IAA in the YEM broth medium, was identified as a potential bio-inoculant. This is the first report of IAA production in Bacillus pumilus DS5.

MATERIALS AND METHODS

Isolation of Bacteria

Isolate starch-degrading microorganisms, a representative soil sample weighing one gram was suspended in 9 ml of sterile distilled water and shaken for 10 minutes. The serial dilution plate technique was used to isolate microorganisms from the collected samples using Starch Agar Media (SAM). To prepare sample dilutions up to 10-7, sterilized water was used. The plates were incubated at 35ºC for 24 to 48 hours, and then flooded with 1% iodine reagent and left for 10 minutes. Colonies with good growth were picked and maintained on starch agar slants at 4ºC, and further assessed for enzyme production in liquid medium. The preliminary characterization and identification of the isolate was done using Bergey's Manual of Determinative Bacteriology. Pure cultures were maintained at 4ºC for future use. The entire process was carried out in triplicate.

Identification of bacteria by 16 S rRNA sequencing DNA extraction and PCR Amplification

The genomic DNA of the given organism was isolated according to the following procedure: A single bacterial colony was inoculated in 50 ml LB broth and grown until an absorbance of 600 nm of 0.5-1.0 was reached, and cells were collected through centrifugation at 5000 rpm, at 4°C, for 10 min. The 16S rRNA gene was amplified using universal primers

Bioinformatics analysis

The sequences were compared to the non-redundant NCBI database using BLAST, with default settings used to find the most similar sequence and sorted by the E score. A representative sequence of the ten most similar neighbors was aligned using CLUSTAL W2 for multiple alignments with default settings. The multiple alignment file was then used to create a phylogram using MEGA 5 software.

Production of Indole acetic acid

The production of IAA was detected as described by Ram et al. (2018), with slight modifications. Bacterial cultures were grown for 48 h of incubation at 35°C and pH 7.0. Fully grown cultures were centrifuged at 3000 rpm for 30 min, and the supernatant (2 ml) was mixed with two drops of ortho phosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M FeCl3 solution). The development of a pink color indicates IAA production. The amount of IAA produced was calculated using the standard graph of authentic IAA (Gordon and Weber, 1951).

Optimization for IAA production by *Bacillus pumilus* DS5

Different factors, including incubation period, L-tryptophan concentration, pH, carbon, and nitrogen sources, affected IAA production. The optimization methods and procedures were followed by Kranthi Kumar and Raghu Ram (2016).

Effect of incubation period on IAA production by *Bacillus pumilus* DS5

For the production of indole acetic acid, different incubation periods (12, 24, 36, 48, and 60 h) were maintained in the production medium. IAA was estimated using a spectrophotometer at 540 nm

Effect of L-Tryptophan Concentration on IAA production by *Bacillus pumilus* DS5

Different concentrations of L-Tryptophan (50, 100, 150, 200, and 250 mg) were added to the production medium after sterilization and inoculated with B. pumilus DS5, and incubated for 48 h (optimum time for maximum IAA production) on a rotary shaker at 200 rpm at room temperature. Growth and IAA were measured using a spectrophotometer at 540 nm.

Effect of Carbon sources on IAA production by *Bacillus pumilus* DS5

In the production medium, starch was replaced with 5 different carbon sources (Mannitol, glucose, lactose, sucrose, and maltose) at 1% concentration inoculated with B. pumilus DS5 and incubated for 48 h on a rotary shaker at 200 rpm at room temperature. Control was maintained without a carbon source. Growth and IAA production were measured using a spectrophotometer at 540 nm.

Effect of Nitrogen sources on IAA production by *Bacillus pumilus* DS5

In the production medium, sodium nitrate was replaced with different nitrogen sources (Ammonium sulphate, Potassium chloride, L-Asperagine, Peptone and Beef extract) at 0.5 % level along with L-Tryptophan. Growth and IAA was measured by using spectrophotometer at 540 nm.

Effect of Vitamin sources on IAA production by *Bacillus pumilus* DS5

Different vitamin sources (Vitamin B2, Vitamin B6, Vitamin B12, Thiamine-HCl, Riboflavin, Biotin and Ascorbic acid) were introduced in to the production media and the IAA production was determined by using spectrophotometer at 540 nm.

Statistical Analysis

Three replicates were maintained for each treatment. Statistical analysis of the data was performed using SPSS software (version 20). ANOVA and Duncan's multiple test were carried out as per the data and results were considered to be significant at $P<0.05$.

RESULTS

Form the results the bacterial strains isolated from agricultural fields soils of Andhra Pradesh. Morphological biochemical studies were conducted**.** The present strain was identified with 16S rDNA analysis. Phylogenetic tree constructed with clustal Fig-1.

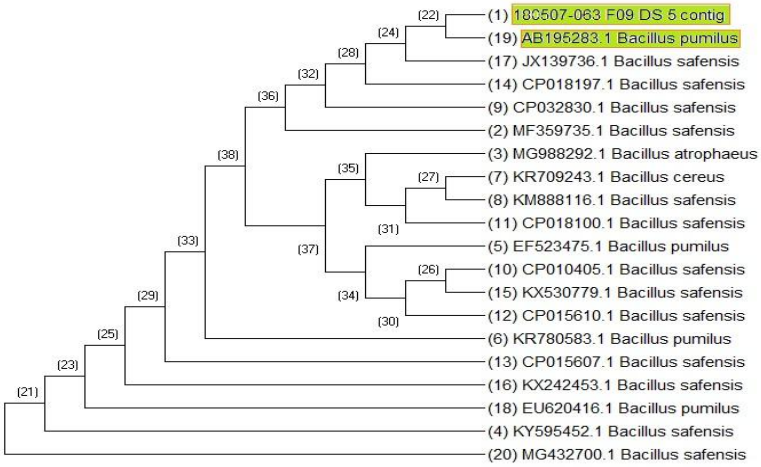

Figure 1: Phylogenetic tree based on 16S rRNA sequences of the genus *Bacillus* obtained from BLAST search showing the position of isolate (ANU-MCB-DS5) and related strains.

Effect of Incubation Period

The results showed that the highest IAA production (105 µg/ml) was observed after 48 hours of incubation period. The strain exhibited initial IAA production at 12 hours of incubation time and increased with prolonged incubation up to 48 hours. However, IAA production decreased after 48 hours of incubation period (Table-1). In a study by Silpa et al. (2018), Bacillus licheniformis DS3 exhibited the maximum IAA production (142 µg/ml) at 0.1% L-tryptophan concentration and after 48 hours of incubation.

Table 1: Effect of incubation period on IAA production

Incubation periods (Hours)	IAA production (µg/ml)
12	22
24	56
36	88
48	105
60	42

The F- Value for incubation period and interactions are all significant with $p<0.05$.

Effect of L-tryptophan concentration

The production of IAA was observed to be dependent on the concentration of L-tryptophan in the production medium, with the optimal concentration ranging from 50 to 250 mg/g (Table-2). IAA production increased with increasing L-tryptophan concentration up

to 200 mg/g, with a maximum IAA production of 240 µg/ml. However, when the L-tryptophan concentration in the production medium was increased beyond 200 mg/g, IAA production decreased (Kranthi kumar and Raghu ram, 2016). This finding is consistent with the report of Swain et al. (2007), who also observed that Bacillus pumilus spp. produced IAA in a tryptophan-dependent manner. It has also been reported that several other bacteria synthesize IAA in a tryptophan-dependent manner (Patten and Glick, 2002).

Table 2: Effect of L-tryptophan concentration on IAA production

L-tryptophan concentration (mg/g)	IAA production (µg/ml)
50	72
100	105
150	180
200	240
250	147

The F- Value for incubation period and interactions are all significant with $p<0.05$.

Effect of pH

The production of IAA was evaluated at different pH levels (5.0, 6.0, 7.0, 8.0, and 9.0). The results indicated that the maximum production of IAA (105 µg/ml) was observed at neutral pH. Conversely, IAA production decreased below and above the neutral pH range. Interestingly, IAA production was also observed at an acidic pH of 5.0 (28 µg/ml). Similar results have been reported for Bacillus, rhizobia, and Trichoderma strains, which showed IAA production at both acidic and alkaline pH (Kumar and Ram, 2012; Ram et al., 2018; Silpa et al., 2018). Moreover, the present strain exhibited maximum IAA production at neutral pH, and slight growth was observed even at alkaline pH (Table-3).

Table 3: Effect of pH

pH	IAA production (µg/ml)
5.0	28
6.0	65
7.0	105
8.0	56
9.0	18

The F- Value for incubation period and interactions are all significant with $p<0.05$.

Effect of carbon and Nitrogen sources

The effect of different carbon sources on IAA production by Bacillus pumilus strain DS5 was investigated using five different carbon sources, namely sucrose, mannitol, glucose, maltose, and lactose. The study also examined the influence of various nitrogen sources in the medium for the production of IAA. The maximum IAA production was observed in the presence of mannitol and ammonium sulfate as carbon and nitrogen sources, respectively (Table 5 and 6). However, the present strain also produced a substantial amount of IAA in the presence of other carbon sources such as sucrose and glucose. The nitrogen source ammonium sulfate was found to support maximum production of IAA in the medium by Bacillus pumilus DS5. Similar reports on Bacillus and rhizobium strains have also shown IAA production in the presence of mannitol and ammonium sulfate as carbon and nitrogen sources (Kumar and Ram, 2012; Ram et al., 2018; Silpa et al., 2018).

Table 4: Effect of carbon sources on IAA production

Carbon sources	IAA production (µg/ml)
Control	12
Sucrose	95
Mannitol	105
Glucose	98
Maltose	82
Lactose	70

*The F- Value for carbon sources and interactions are all significant with $p<0.05$ and at $p<0.01$.

Table 5: Effect of nitrogen sources on IAA production

Nitrogen sources	IAA production (µg/ml)
Control	12
Potassium chloride	80
L- Aspargine	75
Ammonium sulphate	98
Peptone	102
Beef extract	86

*The F- Value for nitrogen sources and interactions are all significant with $p<0.05$ and at $p<0.01$.

Effect of vitamins

Different vitamin sources (Vitamin B2, Vitamin B6, Vitamin B12, Thiamine-HCl, Riboflavin, Biotin and Ascorbic acid) were incorporated into the production medium. Growth of the bacteria was observed and the maximum is in the presence of Biotin 2.95.

Table 6: Effect of different vitamin sources on IAA production

Vitamins	Growth (OD at 540 nm)	IAA production (µg/ml)
Vitamin B2	**1.65**	**58**
Vitamin B6	**1.39**	**42**
Vitamin B12	**2.35**	**68**
Thiamine-Hcl	**2.80**	**80**
Riboflavin	**2.88**	**95**
Biotin	**2.95**	**110**
Ascorbic acid	**1.80**	**55**

The F- Value for incubation period and interactions are all significant with $p<0.05$.

DISCUSSION

According to Srinivas et al. (2002), anoxygenic phototrophic bacteria can produce Indole acetic acid under different cultural conditions. L-tryptophan was found to be necessary for IAA production, although some bacteria were capable of producing IAA without it (Lee et al., 2004; Jayaprakashvel et al., 2014). The production of IAA by B. pumilus DS5 was greatly influenced by the carbon and nitrogen sources used. The choice of carbon source is an important factor to consider when selecting plant growth promoting bacteria. Mohite (2013) reported that glucose was the most suitable carbon source for IAA production in Bacillus megaterium br1, which was isolated from the banana rhizosphere. Suitable nitrogen sources were also applied to different types of isolates, such as NaNO3 for br1 and KNO3 and peptone for Lactobacillus casei br2, which was also isolated from the banana rhizosphere (Mohite, 2013).

CONCLUSION

The current study demonstrated significant variations in the Indole acetic acid production by the plant growth promoting Bacillus pumilus. Optimal IAA production was achieved after a 48-hour incubation period, with 0.1% L-tryptophan concentration and at pH 7.0. Remarkably, we identified for the first time that different vitamin sources have a considerable impact on IAA production and growth of Bacillus pumilus DS5. This study also highlighted the influence of cultural conditions and substrate availability on IAA production by Bacillus pumilus DS5.

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